

COORDINATED GENE EXPRESSION BETWEEN
LONGISSIMUS DORSI MUSCLE AND
INTRAMUSCULAR FAT IN GRAZING BEEF CATTLE

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Abstract: Previous research indicates that metabolism and fiber type of skeletal muscle is related to intramuscular lipid content. The objective of this study was to determine differences in the metabolism and intercellular signaling of skeletal muscle fibers within the same muscle group that could be responsible for the initiation of intramuscular adipose tissue development and differentiation. Longissimus dorsi muscle (LM) samples were collected from steers (n = 12; 385 days of age; 378 kg) grazing wheat pasture. LD samples were dissected under magnification and sorted into 3 categories based on visual stage of development: immature (MM), intermediate (ME) and mature (MA) intramuscular fat (IM). In addition, muscle fibers lying adjacent to each IM category and those not associated with IM tissue were collected and stored separately. Quantitative RT-PCR was used to determine relative fold change in genes involved in metabolism, angiogenesis, formation of extracellular matrix, and intercellular signaling pathways in both LD and IM samples. Gene expression data were analyzed using a general linear model that included the fixed effect of tissue. Pearson correlation coefficients were also computed between gene expression in LD and IM tissue samples that were at the same stage of development. Fatty acid binding protein 4 and *PPAR γ* expression were greater ($P < 0.05$) in more mature IM while *PREF-1* expression was less ($P < 0.01$) indicating successful separation into different maturity categories. Genes associated with metabolism and angiogenesis in LD tissue showed no differences among stages of development. Myostatin expression did not change in LD tissue; however, myostatin receptor and follistatin expression decreased ($P < 0.01$) as IM matured. Collagen type I and VI had evaluated mRNA expression in the skeletal muscle associated with immature IM development. Angiogenic growth factors in MM IM tissue had a strong positive correlation ($r > 0.88$) with angiogenic growth factors in LD associated with MM IM; however, no correlation was observed in ME or MA IM. These data indicate a coordinated effort between LD and IM in early stages of IM development.

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CHAPTER I

INTRODUCTION

The main goal of meat animal production is to produce a high-quality meat product for the customer. Protein growth is the primary concern for producers; however, intramuscular fat is the primary aspect of quality grade in beef cattle. Narrow profit margins in animal agriculture have made improving the efficiency of animal growth an important focus. Producers receive premiums for carcasses with above average intramuscular fat and minimal subcutaneous fat. Therefore, understating the effects that management practices have on feedlot performance and carcass characteristics, like intramuscular fat, could be economically viable for producers. Some studies have shown that the type of growing program has effects on body composition and feedlot performance of beef cattle (Carstens et al., 1991; Drouillard et al., 1991; Hersom et al., 2004) . Data collected from the Vetlife Benchmark Performance data base showed a slight decline in USDA Choice carcasses since 1999, despite an increase USDA Yield Grades of 4 or 5 (Anderson et al., 2007). This data indicates that carcasses are reaching a fatter end point without an increase in quality grade.

Meeting the challenge of increasing meat quality will require a comprehensive knowledge of gene expression changes that occur between muscle and intramuscular fat. Development of intramuscular fat, also called marbling, occurs within the perimysium of muscle bundles. Since marbling develops within close proximity to muscle fibers, it is believed that signaling between myogenic cells and adipocytes may influence marbling development (Kokta et al., 2004).

Intramuscular fat development has been shown to develop closely with capillary networks (Harper and Pethick, 2004). Hausman and Richardson (2004) reported that angiogenesis is an important aspect of adipose tissue development, and that differentiation of adipocytes may be regulated by factors that stimulate the formation of blood vessels also called angiogenesis.

The economic benefits from producing cattle that will receive the USDA quality grade of Choice or better and the weight gain added to cattle during grazing indicate that it is may be advantageous for producers to utilize growing programs to help improve quality grades. To increase the understanding of the physiological mechanisms that regulate the coordinated development of skeletal muscle and intramuscular adipose tissue, we evaluated the effect of rate of gain during the stocker phase on changes in gene expression associated with intercellular signaling mechanisms.

CHAPTER II

REVIEW OF LITERATURE

Growing Programs

Growing programs, commonly referred to as stocker and backgrounding programs in the Great Plains, are composed of a variety of production practices all with one common goal, growth. The stocker or backgrounding phase of production is commonly the time period between weaning of calves and the beginning of the finishing phase. It is estimated that 74 to 82% of yearly calf crop is available as stocker calves to enter a stocker program before entering the finishing stage of production (Peel, 2003). Stocker programs are commonly forage based systems with an emphasis on skeletal growth of the animal rather than fat deposition. Body growth before fattening allows for improved quality and marbling scores because greater maturity is obtained by reaching slaughter finish at a desirable carcass weight (Sainz et al., 1995). Producers often utilize wheat pasture as a grazing program because the profit available from value added to cattle, as weight, along with grain production following removal of the cattle. Grazing stocker cattle can be economically advantageous due to the availability of high quality forage and the favorable seasonality of feeder cattle prices (Horn et al., 2005).

Body composition and feedlot performance are impacted by growing programs. Sainz et al. (1995) reported that during the growing phase cattle frequently are under nutritional stress and when placed on ad libitum feed will undergo compensatory growth. Compensatory growth is a period of more efficient and rapid growth following nutritional and/or environmental stress.

However, the response to nutrient restriction is highly variable among cattle possibly due to genotypic differences or the degree of nutrient restriction (Drouillard et al., 1991). Higher body fat condition doesn't negatively influence feedlot efficiency or performance of calves as previously thought (Hersom et al., 2004; McCurdy et al., 2010). Greater efficiency, due to increased gain and decreased dry matter intake, was observed in cattle placed directly into a feedlot following weaning (Myers et al., 1999). Genetic potential, nutrient availability, and the environment are factors that can affect the composition of growth in beef cattle. Alterations in carcass fat content may be achieved by modification of final finished body weight through nutrition and hormonal factors, even though maximum growth rate is genetically set (Owens et al., 1993). Sainz et al. (1995) suggested that when growth is restricted during the growing phase the greatest impact is on fat accretion, while muscle development remains unimpaired. A reduction in intramuscular fat was shown in cattle that underwent growth depression before entering the feedlot, suggesting that nutritional status during the growth phase is a vital determinate of final intramuscular fat (Pethick et al., 2004). Nonetheless, whenever cattle are marketed at a common endpoint, most commonly determined by 12th-rib fat, there is no significant alteration in carcass composition (McCurdy et al., 2010; Sainz et al., 1995). This indicates that the impact that growth rate during the growing phase has on final carcass composition is small compared to the effects of growth rate during the finishing.

Tissue Development

Growth can be described as the accretion of bone, muscle, and fat. Studies have shown that growth rates of various tissues differ and appear based on the importance of the functions of the body part or tissue starting with skeleton, muscle, and lastly adipose (Berg and Butterfield, 1968; Mc Meekan, 1940). Bone growth is the ultimate determinant of the length of the individual muscles and therefore becomes a major determinant of total muscle mass (Beitz, 1985). Early in life protein and adipose accretion occur simultaneously; however, later in life fat accretion will

surpass that of protein. This pattern occurs because a shift in metabolic priority from protein to fat accretion which occurs when an animal reaches 50-60% of their mature weight (Trenkle and Marple, 1983). Rate of protein accretion declines as the animal matures because the total turnover of protein becomes an increased fraction of total protein synthesis. In addition, there is an increase in energy cost per unit of net protein accretion, resulting in a decrease rate of protein accretion as protein mass increases (Whittemore and Fawcett, 1976). Protein is less energetically efficient than that of fat accretion due to protein having a faster and less efficient turnover rate than adipose tissue (Moloney et al., 1991). Moloney et al. (1991) reported fat accretion as being 1.6 times more efficient than that of protein accretion. A growth curve comparing the relationship between protein and fat mass to empty body weight indicates that protein mass increases in a linear fashion while adipose tissue is more of a quadratic increase, however upper level was only 500 kg (Owens et al., 1995).

Production of new cells can also be explained as growth (Owens et al., 1993). Beitz (1985) stated that the number of cells control the total capacity of its respective tissue. Cell growth can occur by either hyperplasia or hypertrophy. Hyperplasia is cell multiplication which occurs early in life while hypertrophic growth is through cell enlargement, through incorporation of substrates from the environment into the cell that occurs later in life (Owens et al., 1993). Increase in muscle fiber numbers occurs primarily prenatally with only slight postnatal growth (Moloney et al., 1991). A characteristic of a myoblast is their permanent withdrawal from the cell cycle, which prevents the myoblast from proliferating (Allen et al., 1979). Since myoblasts lack the capability to proliferate, postnatal hypertrophy occurs through incorporation of DNA from satellite cells into the muscle fiber. Allen et al. (1979) reported that the most rapid period of muscle growth corresponded with the rapid period of increasing DNA within the muscle cell. The incorporation of DNA occurs by fusion of satellite cells with muscle cells; the capacity of the muscle cell to grow is attributable to a relatively constant amount of muscle cell cytosol present per nucleus (Young et al., 1979). By

limiting the amount of DNA incorporated into the muscle cell, muscle growth could be regulated (Allen et al., 1979).

Adipose tissue growth rate is known to differ between sites of deposition in the order of internal, intermuscular, subcutaneous, and lastly intramuscular (Beitz, 1985). Unlike skeletal muscle, hyperplasia and hypertrophy both occur in adipose tissue postnatally along with prenatal hyperplasia. Adipoblasts, which originate from undifferentiated mesenchymal cells, divide to form preadipocytes. Preadipocytes lack the ability to divide; therefore, they merely accumulate lipid to form mature adipocytes (Beitz, 1985). Intuitively by increasing the number of fat cells present the capacity of the animal to accumulate fat will also be increased. Metabolic activity has been shown to differ between adipose depots. Fatty acid biosynthesis in intramuscular fat incorporates more carbon from glucose when compared to the subcutaneous depot (Smith and Crouse, 1984). Intramuscular fat is known to be a late maturing tissue in animals due to greater deposition rate occurring later in life; however, this does not mean that the accretion rate of intramuscular fat is also late maturing (Pethick et al., 2004). Adipose deposition is five times greater during the finishing phases when compared to deposition rate during growing phase (Lemieux et al., 1990). In contrast, a serial slaughter study of cattle on a high-energy diet indicated that marbling develops at a constant rate throughout the growing period opposed to being late maturing (Bruns et al., 2004). Another serial slaughter study showed that intramuscular fat development increased at a decreasing rate with an increased number of days on feed (Duckett et al., 1993). Pethick et al. (2004) reported that the rate of intramuscular fat as percent of hot carcass weight (HCW) is constant for every 10 kg increase in HCW there is a 0.47% increase in intramuscular fat.

Muscle Fiber Types

It is an accepted notion among researchers that muscle growth capacity is a characteristic that is fixed at birth, since the total number of fibers doesn't change following birth. The number and type of fibers present in bovine muscle is highly variable among sex, breed type, and muscle type (Lefaucheur and Gerrard, 2000). Myosin is the primary structural protein present in the thick filament of the sarcomere and is a determining factor of the contractile speed. Polymorphisms of the myosin heavy chain (MyHC) is the basis for the contractile type of muscle fibers. Slow contractile muscle are involved mainly in posture, while the fast twitch muscles are responsible for production of movement. Succinodehydrogenase, a mitochondrial enzyme, activity is the determinant between oxidative and nonoxidative MyHC isoforms (Lefaucheur and Gerrard, 2000). MyHCs can be categorized as either slow or fast-twitch fibers. Slow-twitch fibers are known as type I fibers and display oxidative metabolism characteristics. Fast-twitch fibers are known as type II fiber and have three major isoforms, IIA, IIB, and IIX. Type IIA exhibit predominantly oxidative metabolism, while IIB primarily glycolytic metabolism. The IIX isoform fibers are intermediate to IIA and IIB exhibiting partial oxidative and partial glycolytic (Allen et al., 2001; Brooke and Kaiser, 1970; Klont et al., 1998). Studies utilizing PCR amplification have shown that bovine muscle does not express the type IIB MyHC isoform (Chikuni et al., 2004; Tanabe et al., 1998). There is an adaption in the energetic metabolism of skeletal muscle during development. Lefaucheur and Gerrard (2000) reported that as a whole, muscle metabolism becomes more glycolytic with increasing age. It has been determined that bovine are more mature at birth than other farm animals due to an earlier start in metabolic differentiation in cattle (Gagnière et al., 2000).

Muscle fiber types are adaptable and can be modified according to management program which can be attributed to differences in plane of nutrition and amount of exercise (Klont et al., 1998). A study comparing bulls grown on a nutrient restricted diet, loose housing management program

to bulls raised on a concentrate diet, tie-stall program showed changes in fiber type between the different management programs. The restricted cattle had skeletal muscle that exhibited a higher number of the more oxidative type I fiber type (Vestergaard et al., 2000). Similarly, a percent increase in type I fiber types was seen in cattle raised in a grazing program when compared to cattle in a grain fed system (Shibata et al., 2009). These studies indicate that muscle metabolism becomes more oxidative with nutrient restricted diets and increasing exercise. Lefaucheur and Gerrard (2000) reported that when induced by physical exercise, the transition of fiber types follows the order IIB, IIX, IIA, I. Management practices aren't the only factor that can influence muscle fiber metabolism; breed and muscle of different function type can also impact muscle fiber metabolism. Comparison of Charolais steers to Angus steers of the same age showed that Charolais cattle had greater fiber area of primarily glycolytic fibers (Johnston et al., 1981). Vestergaard et al. (2000) demonstrated that the different function of muscle will also impact fiber metabolism. They stated that the semitendinosus had the most glycolytic metabolism, while the supraspinatus had the most oxidative metabolism and the longissimus dorsi was intermediate to the two.

MyHC isoforms composition in skeleton muscle are also an important factor in the quality of meat attributed to their effect on post-mortem changes (Klont et al., 1998). Chikuni et al. (2004) indicated that the conversion of muscle to meat through rigor mortis is affected by differences in ATPase activity of MyHC isoforms. Tenderness and meat discoloration are meat quality parameters important to the consumer that can be impacted by post-mortem changes of skeletal muscle. Increase in percentage of oxidative muscle fibers have shown a correlation with increased tenderness, increase in marbling score, and a decrease in shear force (Calkins et al., 1981). Fiber types have also been shown to impact rate of meat discoloration. Higher frequency of glycolytic fibers can be related to paler meat with lower intramuscular fat (Wegner et al., 2000). This may be because with higher proportions of oxidative fibers within a muscle there will also be an

increased concentration of mitochondria. Mitochondria compete with myoglobin for oxygen and cause the muscle to have a darker color (Monin and Ouali, 1991).

Satellite Cells

Skeletal muscle has the ability to adapt according to demands such as growth, training, or injury. The adaptive ability is possible mainly due to a small population of cells called satellite cells (Hawke and Garry, 2001). Satellite cells can be described as cells that are closely associated with the myofiber and reside between the sarcolemma and basal lamina (Mauro, 1961). Satellite cells provide additional myonuclei to growing myofibers. Fusion of satellite cells to myofibers are the only form of postnatal muscle growth because muscle fiber numbers aren't capable of increasing following birth (Schultz, 1996). Mitotically quiescent satellite cells remain in an unperturbed state and are nonproliferative. Quiescent satellite cells will become activated, proliferative and will express myogenic markers in response to a stimuli, such as growth or myotrauma (Hawke and Garry, 2001). Activation of the quiescent satellite cells will allow fusion to form new myofibers or regenerate damaged muscle (Hawke and Garry, 2001). Schultz (1996) reported that some satellite cells withdraw from the cell cycle or cycle slowly to serve as a reserve for satellite cells, allowing for satellite cells to be available for future postnatal growth or repair. The process of self-renewal by quiescent satellite cells allows for the numbers of satellite cells in the population to remain in a constant state (Gibson and Schultz, 1983). Pluripotent stem cells have the capacity to differentiate into various cell types with diverse cells (Harper and Pethick, 2004). A study evaluating the potential of satellite cells to differentiate into different cell lineages besides that of myogenic cells found that adult satellite cells are capable of differentiating into osteocytes and adipocytes following treatment with respective inducer (Asakura et al., 2001). It has also been reported, in a more recent study, that satellite cells are committed to the myogenic lineage and can't undergo permanent fate changes to non-myogenic lineages (Starkey et al., 2011).

Skeletal Muscle and Satellite Cell Gene Expression

Skeletal muscle gene expression in meat producing animals has been shown to be regulated by many growth factors (Bass et al., 1999; Dayton and White, 2008; Hocquette et al., 1998). Growth factors indicated to be of importance in regulation of myoblasts and satellite cell proliferation and differentiation are myostatin, IGF-1, MyoD, and MyoG; these factors can have either an inhibitory or stimulatory effect on skeletal muscle growth. These growth factors have effects on both embryogenesis of myoblasts as well as regulators of the satellite cell population (Hawke and Garry, 2001).

Insulin-like growth factors I and II (IGFs) stimulate the proliferation and differentiation of myoblasts (Bass et al., 1999; Dayton and White, 2008). IGF-1 stimulates growth in both pre and postnatal periods, while IGF-II effect on growth occurs during fetal growth and has no effect on postnatal growth (Collett-Solberg and Cohen, 2000). Dayton and White (2008) reported that stimulation of protein synthesis and suppression of protein degradation are actions of IGF-1 in myogenic cells. IGFs have been shown to be required for normal growth and survival of animals supported by studies that reported an increased death rate and reduction in postnatal growth rate in IGF-1 knockout mice (Baker et al., 1993; Liu et al., 1993). Paracrine and/or autocrine actions of IGF-1 from local production in skeletal muscle may play a pivotal role in supporting muscle growth (Sjögren et al., 1999). Activity of IGFs are regulated by insulin-like growth factor binding proteins 1-6 (IGFBPs). IGFBPs are high affinity binding proteins that have the capability to either enhance or inhibit the ability of IGF-1 to bind with its IGF receptor (IGF1R), which is responsible for much of IGF-1 biological activity (Baxter, 2000). IGFBP-1, -3, and -5 have both stimulatory and inhibitory effects on IGF, while IGFBP-2 and -4 have solely inhibitory effects on IGF action. Tan et al. (2006) used bovine bone marrow stromal cells to study adipogenesis in vitro and reported that IGFBPs -3, -4, and -5 increased during angiogenesis with IGFBP5 having the greatest increase. IGFBP-6 is the only IGFBP that preferentially binds to IGF-II instead of

IGF-I (Collett-Solberg and Cohen, 2000). Dayton and White (2008) also showed that IGFBP-3 and -5 have hindering effects on myostatin activity; however, the mechanism of these processes are poorly understood. In vivo, IGF-1 has been shown to have the same capability to increase proliferation and differentiation in satellite cells as seen in myoblasts (Allen and Boxhorn, 1989).

Myostatin is a negative regulator of muscle growth having inhibitory effects on myoblast development as well as regulatory effects on satellite cell activity in adult skeletal muscle. The negative regulatory actions of myostatin can be observed in the phenotype of double-muscling cattle, who have a naturally occurring mutation in their myostatin gene. This breed of cattle display an increased number of muscle fibers and to an extent an increase in muscle size (Arthur, 1995). Bass et al. (1999) showed that myostatin is expressed in bovine muscle from day 16 of gestation throughout the animal's adult life, in certain muscles. In bovine muscle, myostatin expression is highest during gestation when the formation of muscle fibers is occurring (Oldham et al., 2001). Myostatin also functions as a negative regulator of satellite cell proliferation by regulating satellite cell mitotic activity and self-renewal. This is supported by data that reported that myostatin-null mice display muscle hypertrophy and increased postnatal growth, both of which can be related to satellite cell activity (Grounds and Yablonka-Reuveni, 1993).

McCroskery et al. (2003) showed that satellite cells do express myostatin. They reported an increase in satellite cells per unit length of muscle fiber in mice that lacked myostatin indicating that there is an increase in self-renewal of satellite cells in the absence of myostatin. The effect of myostatin on satellite cell quiescence is supported by higher percent of satellite cells in the activated state in myostatin-null mice compared to wild type mice (McCroskery et al., 2003).

Binding of myostatin with activin type II receptors (ACVR2B) causes signaling that negatively influences muscle growth (Lee et al., 2005). A study utilizing transgenic mice that overexpress the propeptide follistatin showed that overexpression of follistatin resulted in a phenotype similar to a myostatin-null mouse (Lee and McPherron, 2001). Another study using follistatin-mice

resulted in mice with decreased muscle mass after birth, compared to control mice, which would be an expected phenotype with over activity of myostatin (Matzuk et al., 1995). These studies show that follistatin negatively regulates the activity of myostatin.

The MyoD family of transcription factors (MRFs) including MyoD, myogenin (MyoG) and Myf5 are important factors involved in muscle growth. Myf5 and MyoD are responsible for the determination of myogenic cells and myoblast formation during embryogenesis. Activation of muscle differentiation is a function of MyoG (Hawke and Garry, 2001). MyoD and MyoG bind to the same promoters but have distinctly different roles in skeletal muscle gene expression. MyoD, independent of MyoG activity, is sufficient to initiate expression of early genes, but to activate late genes combined activity with MyoG is necessary. MyoG does not efficiently activate muscle genes without synergistic activity with MyoD on genes expressed late and responsible for myogenic differentiation (Cao et al., 2006). Despite the understanding of the role of MRFs in embryogenesis, there are a lack of studies that aid in clarifying the roles of MRFs in the growth of adult skeletal muscle. MRFs have also been shown to be isolated according to fiber type. Studies support that MyoD is localized in fast-twitch fibers while MyoG is localized in slow-twitch myofibers (Hughes et al., 1993; Seward et al., 2001).

Paired box transcription factor (Pax7) expression has been shown to be specific to quiescent satellite cells (Hawke and Garry, 2001; Seale et al., 2000). Seale et al. (2000) demonstrated that Pax7-null mice exhibited decreased muscle mass, likely attributed to deficient postnatal skeletal muscle growth. Lack of postnatal growth is caused by the absence of the formation of muscle satellite cells in mice lacking Pax7 expression (Seale et al., 2000). Two MRFs, MyoD and Myf5, also may be used as satellite cell markers along with Pax7. MyoD and Myf5 are not expressed in quiescent satellite cells; however, they become expressed once satellite cells become activated (Cornelison and Wold, 1997). Studies have determined that MyoD expression isn't detectable until 24 hours after satellite cell activation and Myf5 expression isn't detectable until 48 hours

following activation (Cornelison and Wold, 1997; Smith et al., 1994). These studies indicate that MyoD and Myf5 can be used as indicators of satellite cell activation and proliferation.

Extracellular Matrix

The extracellular matrix (ECM) has multiple functions that it performs within the body. The ECM provides anchorage and structural support for cells, separates one tissue from another, as well as regulating intercellular communication between tissues (Hausman, 2012; Velleman, 2012). A study indicated that the formation of functional skeletal muscle requires the presence of ECM, so muscle growth can be patterned (Purslow and Duance, 1990). Interactions of cells with ECM is important because ECM components provide chemical information to cells. Hausman (2012) reported that cellular functions that require growth factor mediation for activation can occur within the ECM due to the rapid and local availability of growth factors. Growth factors are locally available because ECM can act as depot for growth factors that have acquired and stored within the ECM. ECM in skeletal muscle has three layers of connective tissue: endomysium, perimysium, and epimysium with each layer differing in composition of collagen types present in the ECM (Purslow, 2002; Purslow et al., 2012). The perimysium which surrounds muscle fibre bundles is important when concerning intramuscular fat because the perimysium is where intramuscular fat development occurs. ECM of skeletal muscle have both marbling and connective tissue present, the intramuscular adipocytes are embedded within the connective tissue (Du and Carlin, 2012). Collagens, found exclusively in the ECM, have differing physiological and development functions within various cell types (Nakajima et al., 2002). In a study using a bovine intramuscular preadipocytes (BIP) cell line Nakajima et al. (1998) reported that BIP cells had the ability to produce collagens type I, III, IV, with VI being the most highly expressed collagen. Kubo et al. (2000) reported that the same collagen types stated by Nakajima et al (1998) are what compose the fibrous network of interstitial collagens during the late stage of adipocyte differentiation. Collagen type I (COL1) is the major component of the anchoring system that

connects adipocytes to each other and the fat clusters to the basement membrane (Kubo et al., 2000). Collagen VI (COL6) appears to be specific for adipocytes because it the primary collagen expressed by adipocytes (Mariman and Wang, 2010; Nakajima et al., 2002; Nakajima et al., 1998). An in vivo study using BIP cells showed that COL6 was capable of increasing lipid synthesis (Nakajima et al., 2002). The same authors also speculated that COL6 may be associated with the increase intramuscular fat development associated with Japanese Black cattle. The level of COL6 expression in the perimysium and endomysium ECM of Japanese Black are more abundant than other cattle breeds (Nakajima et al., 2002; Nakajima et al., 1998). Mariman and Wang (2010) showed changes in expression levels of collagens and differences were seen among collagen types as adipocytes differentiated. COL1 expression levels decreased early during differentiation but later increased; COL6 expression pattern was the opposite having high level early during differentiation and declining late.

ECM is constantly being remodeled due to the high degree of replacement that occurs within collagen. Changes in the ECM components are associated with remodeling. Remodeling must occur for the changes associated with development IM depots. Balance between the activities of constructive and destructive enzymes is essential for maintenance of the ECM homeostasis (Mariman and Wang, 2010). In vivo studies have shown that MMP2 and -9 are released by adipocytes during differentiation (Bouloumie et al., 2001). The same study also demonstrated that MMP2 and -9 play a role in differentiation through the modulation of components within the ECM; MMP2 expression constantly increased with differentiation, while MMP9 was shown to have a strong downregulation at the end of differentiation period. Bouloumie et al. (2001) also reported that MMP2 and -9 are essential for the degradation of ECM associated is angiogenesis. MMPs are secreted as zymogens and are activated by the proteolytic removal of a small domain that shields the active site of the enzyme (Purslow et al., 2012). Tissue inhibitors of metalloproteinases (TIMPs) regulate the activity of MMPs within the ECM (Mariman and Wang,

2010; Purslow et al., 2012). Four TIMPs have been discovered however only one, TIMP4, has specificity for adipose tissue (Greene et al., 1996). Studies have shown that TIMP4 has binding affinity for both MMP2 and -9 (Melendez-Zajgla et al., 2008). The effect of both MMPs and TIMPs are essential for remodeling to occur.

Angiogenesis

Vasculature and the growth rate of tissue like muscle and adipose are interrelated. Studies have shown that there are variations in capillary density of the microvasculature within different muscle types. These variations are due to changes in composition, metabolic capabilities, and response to exercise of myofibers (Cherwek et al., 2000; Jensen et al., 2004; Williams and Annex, 2004). Vestergaard et al. (2000) stated that muscle with more Type I (oxidative) muscle fibers had significantly greater capillary density. Harper and Pethick (2004) reported that, when observed under magnification, it was seen that IM fat developed within close proximity to blood capillary networks. Besides delivery of nutrients to tissues, deposition and mobilization of adipose tissue are dependent upon blood circulation (Ballard, 1978). Studies, utilizing moderate-framed continental beef cattle, showed that the areas of muscle containing IM adipose tissue and increased vasculature tended to have higher prevalence of muscle fibers that exhibit oxidative metabolism (Melton et al., 1974; Melton et al., 1975). Cattle are fed higher concentrate diets to increase IM fat deposition. Interestingly, oxidative metabolism which favors angiogenesis and IM deposition is more prevalent in cattle fed high roughage diets (Vestergaard et al., 2000). In human skeletal muscle, satellite cells have been shown to be associated more with capillaries than myonuclei (Christov et al., 2007).

Each adipocyte is surrounded by a capillary network making adipose tissue growth dependent upon growth and formation of new capillaries. The ability of intramuscular fat depots to continue to grow throughout an animal's life suggests that adipocytes are capable of recruiting new

capillaries (Rupnick et al., 2002). Angiogenic remodeling is the process of modification of existing vascular network, whereas angiogenic sprouting is the formation of new vessels from existing vessels into area that were previously avascular (Yancopoulos et al., 2000). Numerous vascular specific growth factors, including vascular endothelial growth factor (VEGF) and angiopoietins (Ang1 and-2), influence vascular remodeling. VEGF is the principal vascular specific growth factor needed to initiate immature vascular formation, whether it be through vasculogenesis during development or angiogenic sprouting in adult tissue. (Hausman and Richardson, 2004; Papetti and Herman, 2002; Yancopoulos et al., 2000). Disruption of a single VEGF allele results in severe abnormalities in vascular development which leads to embryonic death (Carmeliet et al., 1996). VEGF doesn't affect angiogenesis only in fat depots. In skeletal muscle, VEGF acts as a signal for the remodeling of myocytes as well as angiogenesis. Acting as a signal allows for capillary density to match that of the oxidative metabolism capacity of myofibers (van Weel et al., 2004). Ang1 is responsible for the stability of blood vessels walls, while Ang2 is antagonistic to Ang1 destabilizing the walls of the blood vessels (Harper and Pethick, 2004; Yancopoulos et al., 2000). Studies performed by Yancopoulos et al. (2000) reported that deficiency in Ang1 resulted in impaired angiogenic remodeling but normal development of primary vasculature. The same study showed that overexpression of Ang1 and -2 resulted in lethal vascular defects. Rupnick et al. (2002) demonstrated that angiogenesis could be a means of adipose tissue regulation through treatment of mice with anti-angiogenic factors that resulted in the decreased weight of fat pad by 12 to 22% along with a decrease in total body weight.

The breakdown and degradation of connective tissue is an important characteristic essential for angiogenesis especially angiogenic sprouting (Yancopoulos et al., 2000). Studies of fetal pigs adipose tissue show that in capillary beds present in dense connective tissue are immature and will have few adipocytes, while loose connective tissue allows for mature capillary beds that

contain more adipocytes (Hausman and Thomas, 1984). Another study evaluating adipose tissue of pigs found that dense connective tissue observed in fetal adipose tissue was not present in adipose tissue of growing pigs (Hausman and Kauffman, 1986). The results indicate that breakdown of connective tissue is critical for the elaboration of capillaries necessary for mature adipocyte development.

Regulation of Adipogenesis

The first step of adipogenesis is differentiation a process that commits progenitor cells to the adipocyte lineage termed adipoblasts. Once committed adipoblasts undergo exponential replication until they reach confluence. Early markers of differentiation are expressed in cells now called preadipocytes that further proliferate. Once proliferation of preadipocytes is finished markers of late differentiation are expressed causing cells, now called adipocytes, to accumulate fat displacing the nucleus (Boone et al., 2000; Kokta et al., 2004). Differentiation of preadipocytes to adipocytes leads to changes in morphology as well as gene expression (Kokta et al., 2004). IGF-I has been confirmed to positively influence preadipocytes proliferation and differentiation (Smith et al., 1988). However, growth hormone is necessary to initiate the paracrine/autocrine action needed for the secretion of IGF-I (Gregoire et al., 1998). Preadipocyte factor 1 (Pref-1) functions to maintain preadipocytes by inhibiting their differentiation into adipocytes (Smas and Sul, 1993). Smas and Sul (1993) also reported that Pref-1 expression is abundant in preadipocytes but is absent in adipocytes. Peroxisome proliferator activated receptor- γ (PPAR γ) is essential for the differentiation of preadipocytes as well as the maintenance of the differentiated state (Rosen and MacDougald, 2006). Fibroblast were induced to differentiate into adipocytes when expression of PPAR γ was forced (Tontonoz et al., 1994). Rosen and MacDougald (2006) state that without expression of PPAR γ promotion of adipogenesis doesn't occur. Fatty acid binding proteins (FABPs) and fatty acid synthase (FASN) are expressed during late differentiation and can be used as makers for mature adipocytes. Synthesis of saturated fatty

acids is the result of FASN (Schmid et al., 2005) , while FABPs is responsible for transportation of fatty acids into adipocytes (Frühbeck et al., 2001a) According to Jeong et. al (2012), fatty acid availability influence the amount of intramuscular fat development and an increase in uptake of fatty acid into muscle cells contributed to increased intramuscular fat development.

Adipose tissue has been shown to be an endocrine organ that produces factors including leptin, adiponectin, and resistin (Miner, 2004). Production of these adipokines are proportional to the size of the fat mass (Argiles et al., 2005). Leptin is an adipokine whose primary role is regulation of energy (Houseknecht et al., 1998). Leptin can also be used as an indicator of energy balance due to an increased expression in response to feed and reduced expression during fasting or insulin deficiency (Fehmann et al., 1997). Decrease in energy conservation, increased body energy utilization and partitioning of substrates towards oxidation than storage are all results of actions of leptin in the periphery (Margetic et al., 2002). Prevention of internalization of lipids in adipocytes could be an autocrine function of leptin synthesized in adipocytes (Frühbeck et al., 2001b). Adiponectin is another adipokine secreted by adipose tissue that has a role in energy homeostasis regulation as a result of its insulin-sensitizing effects (Fang and Sweeney, 2006). Regulation of energy homeostasis is a product of adiponectins ability to improve glucose metabolism, stimulate fatty acid oxidation, and decrease plasma triglycerides (Beltowski, 2003). Two receptors for adiponectin (Adipor) have been identified; Adipor1 has been shown to be expressed primarily in skeletal muscle, while Adipor2 is expressed predominantly in the liver (Ahima, 2006; Blüher et al., 2006). Knockout of the adiponectin gene in mice models produced mice with severe insulin resistance along with increased lipid deposition within the muscle when they were fed a high fat diet (Maeda et al., 2002). The actions of the adipokine resistin contrasts to those of adiponectin because resistin increases the insulin resistance of tissues (Meier and Gressner, 2004).

Extracellular signals can also impact the differentiation of adipose tissue. Some WNT family proteins (WNT10b and WNT5b) have been shown to influence development of adipocytes through paracrine and autocrine mechanism (Rosen and MacDougald, 2006). In vivo studies have shown that the inhibitory effects of WNT protein on the differentiation of adipocytes is a result of WNT proteins blocking the expression of PPAR γ and C/EBP α (Bennett et al., 2002). According to Rosen and MacDougald (2006), WNT10b expression is the primary WNT protein responsible for the inhibition of adipocyte differentiation. Conversely, WNT5b is responsible for promoting differentiation and expression is induced during adipogenesis (Kanazawa et al., 2005). The differentiation of adipocytes is a complex process influenced by both paracrine/autocrine growth factors and extracellular signals.

Intercellular Signaling

Research has shown that interactions between myogenic cell and adipocytes play a significant role in the rate and extent of adipogenesis, myogenesis, lipogenesis, and lipolysis (Kokta et al., 2004). Balance between uptake, synthesis, and degradation of triglycerides is essential for accumulations of fat within the muscle to occur (Hocquette et al., 2003). Since intramuscular fat develops in close proximity to muscle fibers, the paracrine activity between skeletal muscle and adipose tissue of factors like IGF-1, leptin, and adiponectin have important implications on the development of muscle and adipose tissue along with influences on energy utilization (Kokta et al., 2004). Lipid partitioning in skeletal muscle has been altered by leptin through an increase in muscle fatty acid oxidation and decreased incorporation of fatty acids into triglycerides (Muoio et al., 1997). Adiponectin had the same effect on skeletal muscle as it increased fatty acid oxidation (Scherer et al., 1995). In vivo studies have determined that glucose uptake is inhibited in skeletal muscle cell cultures when resistin is present (Moon et al., 2003). The addition of resistin to myoblast in culture demonstrates inhibition of differentiation (Kim et al., 2001).

Conclusion

Growing programs for beef cattle are valuable programs for altering frame size and tissue deposition to target for specific markets. Adjusting the plane of nutrition during growing periods can affect both skeletal muscle and intramuscular fat development. The literature suggests that growing programs do not significantly affect final carcass composition of beef cattle. However, the changes in muscle fiber type and the activity of satellite cells, as a result of growing programs, may affect the extent of angiogenesis and intramuscular fat deposition during the finishing phase resulting in changes in carcass quality. Understanding how development of skeletal muscle and intramuscular fat are interrelated is essential to help develop methods to efficiently meet the consumers demand for high quality beef.

CHAPTER III

MATERIALS AND METHODS

Animals and Treatments

Twelve Angus steers from the Range Cow Research Center-South Range Unit near Stillwater, OK were used for this study. Details regarding cattle, cattle and pasture management and treatments were reported by Sharman et al. (2013).

Tissue Collection

Steers were harvested at the Food and Agricultural Products Center (FAPC) and longissimus dorsi muscle (LM) samples were collected as described by Hersom et al. (2004). Tissue samples used were from steers harvested in the first intermediate harvest by Sharman (2012). Tissue samples were stored at -80°C in RNAlater (Invitrogen). Longissimus dorsi samples were dissected, under magnification, according to the maturity of the intramuscular fat. Intramuscular fat was identified in cross-sections of LM samples and sorted, based on visual assessment (Figure 1), into one of three categories: immature (MM), intermediate (ME), and mature (MA). Intramuscular fat was removed from muscle tissue and stored separately according to maturity. Muscle fibers lying immediately adjacent to intramuscular depots was collected and stored separately according to the maturity category of the intramuscular fat it was associated with. Muscle fibers not associated with any intramuscular adipose tissue development were also collected and stored separately.

RNA Extraction

RNA later was removed from both muscle and adipose tissue samples before being pulverized and homogenized in TRIzol reagent (Ambion) for total RNA isolation. RNA was isolated from both muscle and fat samples following the manufacturer's procedures for TRIzol. Following isolations of RNA, a clean-up procedure was used to remove additional fat from the sample and removal of any carry over guanidine isothiocyanate from the TRIzol procedure by adding chloroform (1:1 ratio) to isolated RNA. Mixture was centrifuged at 4°C for 5 minutes at 20,000xg. The upper aqueous phase was removed and transferred to a fresh tube and then phenol:chloroform:isoamyl alcohol (25:24:1) was added in a 1:1 ratio. Mixture was centrifuged at 4°C for 5 minutes at 20,000xg. Upper aqueous phase was transferred to a fresh tube and 100% ethyl alcohol was added (2.5:1 ratio) along with 3 M sodium acetate (0.1:1 ratio) and incubated for 60 minutes at -80°C. Following incubation, the RNA was precipitated and the supernatant was discarded. The RNA pellet was washed with 75% ethyl alcohol and the RNA pellet was allowed to air dry then was resuspended in diethylpyrocarbonate (DEPC) -treated water and stored at -80°C. The quantity of RNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and RNA integrity was determined using gel electrophoresis. Total RNA (1.0µg) was used to synthesize cDNA using a reverse transcription kit (QuantiTect, Qiagen Inc., Valencia, CA). Following reverse transcription and amplification, samples were quantified using a NanoDrop ND-1000 spectrophotometer. cDNA samples were stored at -20°C until gene expression analysis was performed.

qRT-PCR Protocol

Quantitative real-time PCR (qRT-PCR) was used to determine the mRNA expression level of specific genes of interest. Gene specific primers were designed using exonic sequences obtained from the National Center for Biotechnology Information (NCBI) using a Primer3 software

package (Rozen and Skaletsky, 1999). When possible, primers were designed to be intron spanning in order to prevent amplification of contaminating genomic DNA. Specificity of each primer set was evaluated by comparing primer sequences to the database of GenBank using the blast tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Complementary forward and reverse primer sequences for each primer pair were evaluated with the OligoAnalyzer 3.1 (Integrated DNA Technologies, Coralville, IA). A list of primers used in muscle tissue are listed in Table 1 and primers used in intramuscular adipose tissue are listed in Table 2. A Bio-Rad CFX96 real-time detection system (Bio-Rad Laboratories, Hercules, CA) was used to quantify mRNA abundance. For both tissue types, qRT-PCR reactions contained 7 μ L of RT² SYBR Green 2X Super Mix (Qiagen), 0.23 μ L of 25 μ M forward primer, 0.23 μ L of 25 μ M reverse primer, 2.77 μ L of Rnase-free PCR water, and 100 ng of template cDNA. Thermal cycling protocol consisted of 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, optimal annealing temperature (specific to each gene) for 30 seconds, followed by 95°C for 1 min. Thermal cycling protocol was performed using a Bio-Rad C1000 thermal cycler. A melt curve was performed after amplification to verify the specificity of each gene. For each gene, all reactions displayed a single peak melt temperature ($\pm 0.5^\circ\text{C}$) indicating a unique product was produced.

Gene Expression

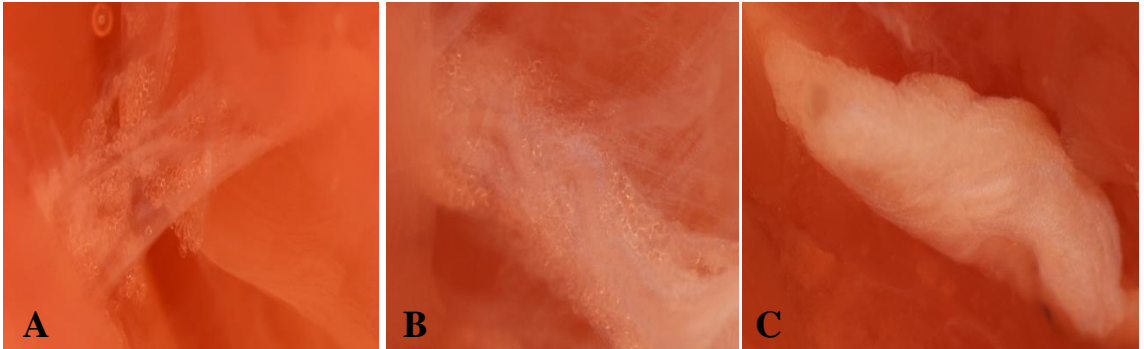
A normalization factor was calculated using geNorm software (Biogazelle., Zwijnaarde, Belgium) by calculating the geometric mean of four reference genes. The genes used as reference genes for muscle tissue were tyrosine 3/tryptophan 5 monooxygenase activation protein zeta (YWHAZ), succinate dehydrogenase subunit A flavoprotein (SDHA), ribosomal protein S9 (RPS9), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For the adipose tissue normalization factor, the reference genes peptidylprolyl isomerase A (PPIA), actin, beta (ACTB), RPS9 and YWHAZ were used. Relative expression for each gene was computed using the $2^{\Delta\text{Ct}}$ method. The threshold cycle (Ct) values for each gene were multiplied by the ratio of the natural

log of 2 to the natural log of the qRT-PCR reaction efficiency factor for each individual gene. Multiplying the Ct value with the ratio of the qRT-PCR reaction efficiency adjusts the Ct value for differences in qRT-PCR efficiency. The relative expression values of the target genes in both muscle and adipose tissue were calculated as $2^{-(\text{normalization factor} - \text{adjusted Target Ct})} \times 10^3$ (Chung and Johnson, 2009). This procedure allowed statistical analysis of linear mRNA expression values after being adjusted for difference in qRT-PCT reaction efficiencies.

Statistical Analysis

mRNA relative expression data was analyzed using SAS (SAS Inst., Inc., Cary, NC). Gene expression data were analyzed using a general linear model (Proc GLM; SAS Inst. Inc., Cary, NC) with year, tissue, treatment, year x tissue, year x treatment, and tissue x treatment interactions as fixed effects in the model. Least Squares means were separated using Fisher Protected LSD with alpha 0.10. Treatment differences were declared significant when $p \leq 0.05$. Pearson's correlations were also computed between the gene expression in muscle and its related intramuscular adipose tissue for each stage of maturity using the Proc Corr procedure of SAS.

Figure 1. Photographs representing the different stages of intramuscular adipose tissue development using a dissecting microscope and camera: A) Immature stage, B) Intermediate stage, and C) Mature stage of development of intramuscular tissue between muscle bundles.



CHAPTER IV

FINDINGS

Results

Muscle Gene Expression

Expression of genes evaluated in LM are reported in Table 3. Year and treatment had no effect on gene expression, so only tissue effects are reported. Genes associated with metabolism, COX3 and ND2, showed no change among different adipose tissue maturity categories. There were no change in fiber type expression among the differing maturity levels of adipose tissue. Satellite cell activity were evaluated in muscle tissue by measuring the expression of PAX7, MYOG, and MYF5. The results show no change in expression in muscle associated with more mature adipose depots indicating that satellite cell activity does not affect intramuscular fat development.

VEGFA, ANGPT1, and ANGPT2 all genes involved in angiogenesis, expression were not different as adipose tissue matured. Extracellular matrix changes were detected with increasing maturity and changes in collagen expression. Levels of COL1A1 ($P = 0.01$), COL1A2 ($P = 0.004$), and COL6A2 ($P = 0.04$) were all increased in muscle associated with immature intramuscular adipose development above the levels of the other maturity categories. However, genes responsible for the remodeling of ECM, MMP2 and TIMP4 remained constant throughout maturity stages. It was attempted to evaluate MMP9 expression; however, mRNA levels were not detectable in skeletal muscle tissue (data not shown).

Intramuscular Adipose Tissue Gene Expression

Expression of genes evaluated in intramuscular adipose tissue are reported in Table 4. Year and treatment had no effect on gene expression so only tissue effects are reported. As intramuscular fat increased with visible maturity, PREF1 levels decreased ($P = 0.002$) while PPAR γ ($P = 0.02$) and FABP4 ($P < 0.0001$) increased with maturity. These results show an increase in the number of differentiated adipocytes indicating that we were successful in separating intramuscular adipose tissue into different stages of maturity based on visual assessment under the microscope. FASN levels didn't change and G3PDH ($P = 0.004$) was lower with more mature adipose tissue, which is contrary to what was expected. Angiogenic genes VEGFA and ANGPT1-2 remained constant as adipose tissue matured. The effects of extracellular signaling pathways on adipogenesis were also evaluated. WNT5B ($P < 0.0001$) mRNA expression in intramuscular adipose tissue was evaluated and had the highest expression in mature intramuscular fat with no differences between immature and intermediate stages of development. It was attempted to evaluate WNT10B but mRNA expression was undetectable in intramuscular adipose tissue.

Intercellular Signaling Pathways Gene Expression

MSTN and IGF1 expression remained constant in muscle tissue regardless of adipose tissue maturity category. Myostatin's receptor, ACVR2B ($P = 0.002$), and FST ($P = 0.01$) measured in intramuscular adipose tissue did differ with maturity being expressed highest in immature adipose tissue. Changes in IGF1 receptor (IGF1R) mRNA expression in adipose tissue were detected with lower expression ($P = 0.03$) in intermediate than immature and mature categories. mRNA expression of IGFBPs were different among adipose tissue, but changes were not consistent between IGFBPs. IGFBP-2 ($P < 0.0001$) and -3 ($P < 0.0001$) both increased with increasing maturity; conversely, IGFBP-6 decreased ($P < 0.0001$) with increasing maturity. mRNA expression of IGFBP-1, -4, and -5 was consistent among maturity categories. Adipokines and their receptors present in muscle were also evaluated. ADIPOQ mRNA expression remained

constant as adipose matured, which is contrary to what was expected. Expression level of the ADIPOQ receptors (ADIPOR) had differing expression patterns. ADIPOR1 expression didn't change with increasing maturity, but ADIPOR2 mRNA expression increased ($P < 0.0001$) as muscle became associated with more mature adipose tissue. As expected, leptin mRNA levels increased ($P = 0.05$) as number of differentiated adipocytes increased. LEP receptor (LEPR) remained constant along maturity categories. An attempt was made to evaluate the resistin signaling pathway; however, resistin mRNA in intramuscular tissue and resistin receptor mRNA in muscle were not detectable (data not shown).

Pearson's Correlations between Muscle and Intramuscular Adipose Tissue

Correlations were calculated between intramuscular adipose tissue and the muscle tissue that corresponded with each maturity category (Table 5). A strong positive correlation ($r = 0.69$ to 0.94) was observed between mRNA expression of VEGFA, ANGPT1 and ANGPT2, genes involved in angiogenesis, in LM and the expression of ANGPT1 and ANGPT2 in intramuscular adipose tissue. mRNA expression of genes involved with adipogenesis measured in adipose tissue, FASN and PPAR γ , were also shown to be strongly correlated ($r = 0.89$ to 0.96) with angiogenic genes measured in muscle tissue. In addition, the adipokines ADIPOQ and LEP showed a moderate to strong correlation ($r = 0.56$ to 0.94) with angiogenic genes mRNA expression in muscle tissue. Interestingly, these correlations were observed only in immature intramuscular development. These data suggest that there is a highly coordinated set of changes that occur between skeletal muscle and intramuscular adipose tissue during the early development of intramuscular adipose tissue.

Discussion

The stage of maturity of adipose tissue resulted in a change in gene expression in both intramuscular adipose tissue and its corresponding muscle tissue. Muscle fiber types are adaptable and can be modified according to differences in plane of nutrition associated with different management programs (Klont et al., 1998). Muscle metabolism can be affected by changes in fiber types because fiber types differ in metabolism characteristics. There was no change in either muscle fiber type mRNA expression or mRNA expression of the metabolic genes ND2 and COX3 related to differing rates of gain. Conversely, Vestergaard et al (2000) reported that when cattle were fed roughage in order to achieve a slower rate of gain there was a greater percentage of oxidative muscle fibers present.

Myostatin mRNA expression in muscle tissue remained constant as intramuscular adipose tissue matured, however, the expression of myostatin receptor, ACVR2B, decreased as intramuscular fat became more mature. A myostatin inhibitor, follistatin, followed the expression pattern of ACVR2B and decreased with increasing maturity of intramuscular fat. Collectively, these data suggest that as intramuscular adipose tissue matures it becomes desensitized to the inhibitory effects of myostatin on the differentiation of adipocytes. IGF-1 mRNA expression in muscle tissue was unchanged as intramuscular fat matured. mRNA expression of IGF-I receptor in adipose tissue was not statistically different; however, numerically it was lower in intermediate than immature and mature categories. The availability and activity of IGF-I would be affected by IGFBPs due to their ability to bind IGF-I, however, the changes in mRNA expression of IGFBPs were not consistent among the IGFBPs. The inconsistency is due to the different effects each IGFBP has on IGF-I activity. IGFBP-2 is known to have solely inhibitory effects on IGF-I actions. The increase in IGFBP-2 in more mature adipose tissue suggests that it may play a role in reducing the proliferative effects of IGF-I on adipocytes allowing them to transition towards lipid accumulation. IGFBP-6 mRNA expression was highest in immature intramuscular fat and

decreased as depots became more mature. IGFBP6 is the only IGFBP that has a higher affinity for IGF-II and preferentially binds with it (Collett-Solberg and Cohen, 2000). Suggesting that IGF-II levels may be elevated in less mature intramuscular fat. Elevated levels of IGF-II was observed in stromal-vascular cells from 75 day old pigs produced IGF-II, but when adipogenesis was induced in these cells IGF-II levels remained steady (Hausman et al., 2002). Both IGFBP-3 and IGFBP-5 have inhibitor and stimulator effects on IGF-I making it difficult to establish the implication of these changes, especially since the mRNA expression pattern of IGFBP-3 is opposite that of IGFBP-5. An in vitro study conducted using bovine bone marrow stromal cells reported an increase in IGFBPs -3,-4, and -5 with -5 having the greatest increase (Tan et al., 2006). The results are contradictory to the results shown in our in vivo study indicating that other extracellular signals may affect IGFBP activity. These changes in IGFBPs mRNA expression throughout the development of intramuscular adipose tissue implies that they may be responsible for the response of intramuscular fat to locally or systemic IGF-I.

It has been well documented that differentiation of adipocytes is controlled by programmed changes in gene expression (Kokta et al., 2004). Changes in intramuscular fat mRNA expression like the decrease in PREF1 with increasing adipose maturity and the increased expression of FABP4 and PPAR γ as adipose matured indicate that the separation of intramuscular fat into maturity categories based on visual assessment was successful. PREF-1 is responsible for maintenance of preadipocytes by inhibiting their differentiation into adipocytes. The pattern of PREF-1 is similar to reports by Smas and Sul (1993) who reported high PREF-1 expression in preadipocytes but absence in adipocytes. PPAR γ was expected to increase as number of differentiated adipocytes became larger due to PPAR γ role in promoting differentiation of adipocytes (Rosen and MacDougald, 2006). Fruhbeck et al. (2001a) indicated that FABP4 is responsible for the transport of fatty acids into adipocytes and FABP4 mRNA expression in intramuscular fat was shown to steadily increase as intramuscular adipose tissue matured

(Pickworth et al., 2011). These results agree with the current study in which FABP was greater in more mature intramuscular depots. The results suggest that FABP4 may be used as a marker for adiposity. FASN which is responsible for synthesis of fatty acids has been shown to have a positive correlation with increasing intramuscular fat content (Jeong et al., 2012). Taken together these results indicate that within a single animal not all intramuscular depots are at the same stage of maturity. Supporting the idea that intramuscular fat development occurs throughout the life time of an animal.

The current study did not show any changes in mRNA expression of genes involved in angiogenesis in either tissue type; however, there was a correlation between angiogenic growth factors in muscle tissue associated with immature intramuscular fat depots and the angiogenic growth factors FASN, PPAR γ , ADIPOQ, and LEP in the corresponding intramuscular fat. These correlations were absent in intermediate and mature categories, indicating that a highly coordinated set of changes occurs between muscle and adipose tissue during the early stage of intramuscular adipose tissue development. The correlations observed is supported by Rupnick et al. (2002) who administered anti-angiogenic agents into obese mice and observed a reduction in fat content over the control mice, concluding that there is a correlation between adipogenesis and angiogenesis. Collectively these data demonstrate that the development of vasculature and adipose tissue are interrelated, especially in early development.

mRNA expression changes in components of the extracellular matrix were observed in muscle tissue associated with increasing maturity of intramuscular adipose tissue. ECM is essential for intramuscular adipose development because adipocytes are embedded with the connective tissue present in the ECM (Du and Carlin, 2012). The current study showed that both collagen type I and VI exhibited the same mRNA expression pattern with muscle associated with immature intramuscular adipose development having the highest expression. On the other hand, a study utilizing murine 3T3-L1 cells reported that the expression levels of collagens were different as

adipocytes differentiated with collagen I expression being decreased early and increasing late in development, while collagen VI expression was the reverse with expression being highest early in development and decreasing late. Tan et al. (2006) reported that in adipogenesis of bovine bone marrow stromal cells, an increase in mRNA expression of COL1A1 and COL1A2 occurred. A microarray study evaluating nutritional restriction effects on gene expression in muscle tissue reported a downregulation of COL1A1 and COL1A2 in nutrient restricted cattle (Byrne et al., 2005). Animals that have a genetic predisposition for high levels of intramuscular fat development have been shown to have increased levels of collagen type I and VI (Nakajima et al., 1998; Wang et al., 2009). These results suggest that the expansion of extracellular matrix is critical for the development of intramuscular adipose tissue, especially during the early stage of adipose development.

Endocrine hormones produced by adipose tissue were shown to have changes in mRNA expression as intramuscular fat matured. Since adipokines are produced by adipocytes, it is logical that adipokine production is proportional to the size of intramuscular adipose tissue mass and would increase as the number of differentiated adipocytes increases (Argiles et al., 2005). Leptin mRNA expression in the current study followed the pattern expected that as the number of differentiated adipocytes increased so did the mRNA expression of leptin. However, the mRNA expression of leptin receptor measured in muscle tissue remained constant in muscle regardless of the maturity of the associated adipose tissue. Another adipokine, adiponectin was numerically higher in more mature adipose tissue; however, it was not statistically different from immature stage of adipose tissue. There was, however, an increase in the mRNA expression of the adiponectin receptor 2 (ADIPOR2) as muscle became associated with more mature intramuscular adipose tissue, yet there was no change in the mRNA expression of ADIPOR1. Interestingly, Ahima (2006) reported that ADIPOR2 is mainly expressed in liver. Whether the increase of ADIPOR2 expression is prompted due to intramuscular development or inherent to muscle fibers

which results in development of intramuscular fat at that location is not clear from these data.

Actions of both leptin and adiponectin have the same result on skeletal muscle which is an increase in fatty acid oxidation and glucose uptake by muscle tissue (Kokta et al., 2004; Muoio et al., 1997), suggesting that muscle closely associated with intramuscular fat development would have improved insulin sensitivity and utilize fatty acids for energy more than skeletal muscle not associated with intramuscular fat development.

Extracellular signaling pathways that influence adipogenesis were also evaluated. WNT5B is responsible for promotion of differentiation of adipocytes (Kanazawa et al., 2005). The current study showed an increase in WNT5B muscle mRNA expression as maturity of intramuscular adipose tissue increased. WNT10B, which has inhibitory effects on adipocyte differentiation, had undetectable levels of mRNA expression in this study. Tan et al. (2006) reported that when adipogenesis was induced in bovine bone marrow stromal cells, WTN10B expression was reduced and undetectable by day 3 post- induction of adipogenesis.

Table 1. Primers used to quantify mRNA expression of genes in muscle tissue using qRT-PCR.

Gene Name ¹	Accession	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product Size, bases	Reaction Eff., %
ND2	NC_006853	AATTCCACCACCACTACCCTGTCA	GAGAGTGGCAAGAATTAGGACGGT	81	96.0
COX3	NC_006853	ACGTCATCATTGGGTCCACCTTCT	GCTTCAAAGCCGAAGTGGTGGTTA	91	91.9
PAX7	XM_002685738	AGAAAGCCAAGCACAGCATC	TCGGGTCTGACTCCACATC		106.2
MYOG	NM_001111325	CTCCCATCGCGCCTCCTG	GCAGATTGTGGGCGTCTG		94.1
MYF5	NM_1741167	CAGCGTCTACTGTCCTGATGT	CTGGAGTTGCAGGTTGAGAA		99.2
MYH1	NM_174117	CCCCTTCTCCCTGATCCACTAC	TTGAGCGGGTCTTTGTTTTTCT		96.5
MYH2	NM_001166227	GCTGCGTCTTCTCACTGGT	CCACCTTCTCTGCTCTGGAT		94.7
MYH7	NM_174727	GGGCAAGAAGAGGAGTGAGG	CGGTAATCAGGATGGACTGG	104	97.0
VEGFA	NM_174216	ACTTCTGCGCTGTTCTCGTTC	CTCTTCCTTCTCTTCTTCCTCCTC	139	99.1
ANGPT1	NM_001076797	GGTCAGAAGAAAGGAGCGAGT	GAATAGCAGCGAGGAAAGCA	98	91.7
ANGPT2	NM_001098855	CTGAGCGGGTGGTTTATTAC	CCGTGCTGAACCTGATACTG	154	89.9
COL1A1	NM_001034039	TGGCAAGAACGGAGATGATG	CCATCCAAACCACTGAAACC	147	101.2
COL1A2	NM_174520	GGCCCAAGTGGAGATAGAGG	AGCAAAGTTCCCGCCAAG	138	97.6
COL6A2	NM_001075126	TCCACGAGAAGCACGAGAG	CCAGGTCGGAGAAGAGTGTC	90	90.1
MMP2	NM_174745	GTCTTCGACGGCATCTCTC	TTCTCCTCCTGTGGGTCTTC	173	82.2
TIMP4	NM_001045871	CCAAATCACCACTGCTATG	TACCCGTAGAGCTTCCGTTC	96	87.1
MSTN	NM_001001525	GTTTGGCTTGCGTTACTCA	TTCTTCTGCTCGCTGTTCT	178	99.1
IGF1	NM_001077828	ATCACATCCTCCTCGCATCT	CTGTCTCCGCACACGAAC	131	98.6
LEPR	NM_001012285	TGGCTTAGAATCCCTTCCTC	TCGGTTTCCCTACTCCTTCC	115	98.8
ADIPOR1	NM_001034055	AAGCACCGGCAGACAAGAG	ATCGTGAAGTGCATGGTAGG	77	98.9
ADIPOR2	NM_001040499	AAGGTCTGGAAGGTCGATG	ATGTTGCCTGTCTCGGTGTG	158	96.10

¹ND2= NADH dehydrogenase subunit 2; COX3=Cytochrome c oxidase subunit III; PAX7= Paired box 7; MYOG= Myogenin; MYF5= Myogenic factor 5; MYH1= Myosin, heavy chain 1; MYH2= Myosin, heavy chain 2; MYH7= Myosin, heavy chain 7; VEGFA= Vascular endothelial growth factor A; ANGPT1= Angiopoietin-1; ANGPT2= Angiopoietin-2; COL1A1= Collagen type I alpha1; COL1A2= Collagen type I alpha 2; COL6A2= Collagen type VI alpha 2; MMP2= Matrix metalloproteinase 2; TIMP4= Tissue metalloproteinase inhibitor 4; MSTN= Myostatin; IGF1= Insulin- like growth factor 1; LEPR= Leptin receptor; ADIPOR1= Adiponectin receptor 1; ADIPOR2= Adiponectin receptor 2.

Table 2. Primers used to quantify mRNA expression of genes in intramuscular adipose tissue using qRT-PCR.

Gene Name ¹	Accession	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product Size, bases	Reaction Eff., %
PREF1	NM_174037	CGACATGACCACCTTCACC	CAGACCGCACAGAGAGACAG	113	80.5
PPAR γ	NM_181024	TTCTCCAGCATTTCCACTCC	GACGCTTTATCCCCACAGAC	233	103.9
FABP4	NM_174314	AGCTGCACTTCTTTCTCACC	TGACACATTCCAGCACCATC	404	96.0
G3PDH	NM_001035354	ATCAATGGAGACAGGCAGAAG	TTTGGAGAGGGACTAGGCAAC	199	90.3
FASN	NM_001012669	AAGCAGGCACACAATATGGAC	TGAAGTCAAAGAAGAAGGAGAGG	244	87.7
VEGFA	NM_174216	ACTTCTGCGCTGTTCTCGTTC	CTCTTCCTTCTCTTCTTCCTCCTC	139	104.1
ANGPT1	NM_001076797	GGTCAGAAGAAAGGAGCGAGT	GAATAGCAGCGAGGAAAGCA	98	108.8
ANGPT2	NM_001098855	CTGAGCGGGTGGTTTATTAC	CCGTGCTGAACCTGATACTG	154	91.3
IGF1R	XM_002696504	ATCCAGGCCACCTCTCTCTC	CCAAGCCTCCCCTATCAAC	142	87.0
IGFBP1	NM_174554	TGCCAGCGAGAACTCTACAA	AGATCCTCTTCCCACTCCAA	193	77.9
IGFBP2	NM_174555	GGACGGGAACGTGAACTTG	GTGCTGCTCCGTGACCTTCT	109	96.5
IGFBP3	NM_174556	TTTCCCACTCAGCCATTC	CAACAAGCCACTCGTCTTCC	152	89.0
IGFBP4	NM_174557	GGAAGGGAAGAGGTCAGAGG	ACAAACGGAGGAGGAAGGAG	164	93.6
IGFBP5	NM_001105327	GAGCAAGCCAAGATCGAAAG	TCTCAGCTCCTCCCACGAAC	190	94.4
IGFBP6	NM_001040495	GCGTACAAGACACTGAGATGG	GGTCACAATTAGGCACGTAGAG	114	90.4
ACVR2B	NM_174495	AACGGCACTACCTCGGACT	ACTCGTGTCTGGGCTTAGA	99	99.5
FST	NM_175801	GAGCTGTGCCCTGAGAGTAA	TCCTCGTCTTCGGTGTCTTC	167	96.0
ADIPOQ	NM_174742	CCATCGCCTCCTACTTCCAC	GGGATCTTCCATGTTGTCCTC	138	93.0
LEP	NM_173928	ACTAGACCGGAGCTGGGATT	GAGGGAATCTTGCTTGATGG	122	99.7
WNT5B	NM_001205628	AGGAGCACATGGCCTACATC	TGCAGGACTCTCCCAAAGAC	127	94.4

¹ PREF1= Preadipocyte factor 1; PPAR γ = Peroxisome proliferator-activated receptor gamma; FABP4= Adipocyte fatty acid binding protein; G3PDH= Glycerol-3-phosphate dehydrogenase; FASN= Fatty acid synthase; VEGFA= Vascular endothelial growth factor; ANGPT1= Angiopoietin-1; ANGPT2= Angiopoietin-2; IGF1R=Insulin-like growth factor 1 receptor; IGFBP1= Insulin-like growth factor binding protein 1; IGFBP2= Insulin-like growth factor binding protein 2; IGFBP3= Insulin-like growth factor binding protein 3; IGFBP4= Insulin-like growth factor binding protein 4; IGFBP5= Insulin-like growth factor binding protein 5; IGFBP6= Insulin-like growth factor binding protein 6; ACVR2B= Activin A receptor IIB; FST= Follistatin; ADIPOQ= Adiponectin; LEP= Leptin; WNT5B= Wingless type MMTV integration site family, member 5B

Table 3. Relative mRNA expression of genes evaluated in longissimus dorsi muscle tissue associated with different intramuscular adipose tissue maturity.

Gene Name ¹	NF ²	MM	ME	MA	SEM	P-value
<i>Metabolism</i>						
ND2	3596.38	2911.58	2769.05	2951.47	309.24	0.25
COX3	34858.58	37546.33	37878.43	37462.86	3527.36	0.92
<i>Satellite Cells Activity</i>						
PAX7	0.19	0.27	0.18	0.19	0.04	0.27
MYOG	32.27	34.22	30.54	33.10	4.21	0.93
MYF5	2.83	3.25	3.26	3.41	0.27	0.49
<i>Fiber Type</i>						
MYH1	4462.69	5386.09	3359.91	3578.03	821.30	0.26
MYH2	14568.91	15925.39	9013.87	10962.19	2609.59	0.20
MYH7	4559.87	5673.68	3754.01	3613.38	785.59	0.21
<i>Angiogenesis</i>						
VEGFA	27.95	41.53	23.58	26.33	7.16	0.27
ANGPT1	11.55	14.37	7.97	10.11	3.27	0.54
ANGPT2	11.85	18.08	10.73	10.70	3.62	0.39
<i>Extracellular Matrix</i>						
COL1A1	18.64 ^b	35.29 ^a	12.42 ^b	11.74 ^b	5.68	0.01
COL1A2	13.85 ^b	23.78 ^a	11.13 ^b	10.62 ^b	2.81	0.004
COL6A2	102.27 ^b	140.68 ^a	106.55 ^b	113.77 ^b	10.25	0.04
MMP2	61.06	69.85	52.25	60.34	5.78	0.18
TIMP4	78.51	76.30	71.36	77.99	5.06	0.72
<i>Intercellular Signaling</i>						
MSTN	8.22	9.08	8.44	8.03	1.146	0.92
IGF1	1.71	1.57	1.49	1.57	0.17	0.83
LEPR	0.33	0.41	0.41	0.39	0.60	0.76
ADIPOR1	30.72	33.03	29.84	30.76	1.70	0.55
ADIPOR2	29.31 ^a	91.56 ^b	122.74 ^c	142.06 ^d	8.01	<0.0001

¹ND2= NADH dehydrogenase subunit 2; COX3=Cytochrome c oxidase subunit III; PAX7= Paired box 7; MYOG= Myogenin; MYF5= Myogenic factor 5; MYH1= Myosin, heavy chain 1; MYH2= Myosin, heavy chain 2; MYH7= Myosin, heavy chain 7; VEGFA= Vascular endothelial growth factor A; ANGPT1= Angiopoietin-1; ANGPT2= Angiopoietin-2; COL1A1= Collagen type I alpha 1; COL1A2= Collagen type I alpha 2; COL6A2= Collagen type VI alpha 2; MMP2= Matrix metalloproteinase 2; TIMP4= Tissue metalloproteinase inhibitor 4; MSTN= Myostatin; IGF1= Insulin-like growth factor 1; LEPR= Leptin receptor; ADIPOR1= Adiponectin receptor 1; ADIPOR2= Adiponectin receptor 2.

²NF= Longissimus muscle not associated with intramuscular fat; MM= Longissimus tissue associated with immature intramuscular fat; ME= Longissimus tissue associated with intermediate intramuscular fat; MA= Longissimus tissue associated with mature intramuscular tissue

^{abcd} LS means differ P<0.05

Table 4. Relative mRNA expression of genes evaluated in intramuscular adipose tissue of different stages of maturity.

Gene Name ¹	MM ²	ME	MA	SEM	P-value
<i>Adipogenesis</i>					
PREF1	164.92 ^a	176.69 ^a	119.57 ^b	10.84	0.002
PPAR γ	5.10 ^b	10.02 ^a	10.07 ^a	1.35	0.02
FABP4	973.13 ^c	4031.96 ^a	2902.08 ^b	428.90	<0.0001
G3PDH	3828.94 ^a	3711.80 ^a	2123.75 ^b	371.43	0.004
FASN	311.31	284.95	356.75	93.71	0.86
<i>Angiogenesis</i>					
VEGFA	9.88	7.94	8.55	0.88	0.30
ANGPT1	0.47	0.34	0.30	0.11	0.52
ANGPT2	40.84	29.23	32.46	3.76	0.09
<i>Intercellular Signaling</i>					
IGF1R	76.14 ^a	49.06 ^b	72.06 ^a	7.39	0.03
IGFBP1	66.89	92.02	60.42	11.42	0.14
IGFBP2	0.97 ^b	0.87 ^b	2.16 ^a	0.15	<0.0001
IGFBP3	320.82 ^b	515.65 ^a	514.45 ^a	32.39	<0.0001
IGFBP4	570.89	623.46	592.14	29.01	0.44
IGFBP5	710.99	508.03	615.94	59.24	0.07
IGFBP6	4622.04 ^a	2376.59 ^b	1988.85 ^b	231.66	<0.0001
ACVR2B	7.17 ^a	5.49 ^b	4.27 ^b	0.52	0.002
FST	172.76 ^a	130.44 ^b	101.82 ^b	15.27	0.01
ADIPOQ	197.33	309.87	319.52	50.65	0.18
LEP	4.58 ^b	10.64 ^a	11.58 ^a	2.09	0.05
<i>Other</i>					
WNT5B	2.32 ^b	2.23 ^b	5.53 ^a	0.38	<0.0001

¹ PREF1= Preadipocyte factor 1; PPAR γ = Peroxisome proliferator-activated receptor gamma; FABP4= Adipocyte fatty acid binding protein; G3PDH= Glycerol-3-phosphate dehydrogenase; FASN= Fatty acid synthase; VEGFA= Vascular endothelial growth factor; ANGPT1= Angiopoietin-1; ANGPT2= Angiopoietin-2; IGF1R=Insulin-like growth factor 1 receptor; IGFBP1= Insulin-like growth factor binding protein 1; IGFBP2= Insulin-like growth factor binding protein 2; IGFBP3= Insulin-like growth factor binding protein 3; IGFBP4= Insulin-like growth factor binding protein 4; IGFBP5= Insulin-like growth factor binding protein 5; IGFBP6= Insulin-like growth factor binding protein 6; ACVR2B= Activin A receptor IIB; FST= Follistatin; ADIPOQ= Adiponectin; LEP= Leptin; WNT5B= Wingless type MMTV integration site family, member 5B

² MM= Immature intramuscular fat; ME= Intermediate intramuscular fat; MA= Mature intramuscular fat

^{abc} LSmeans differ P<0.05

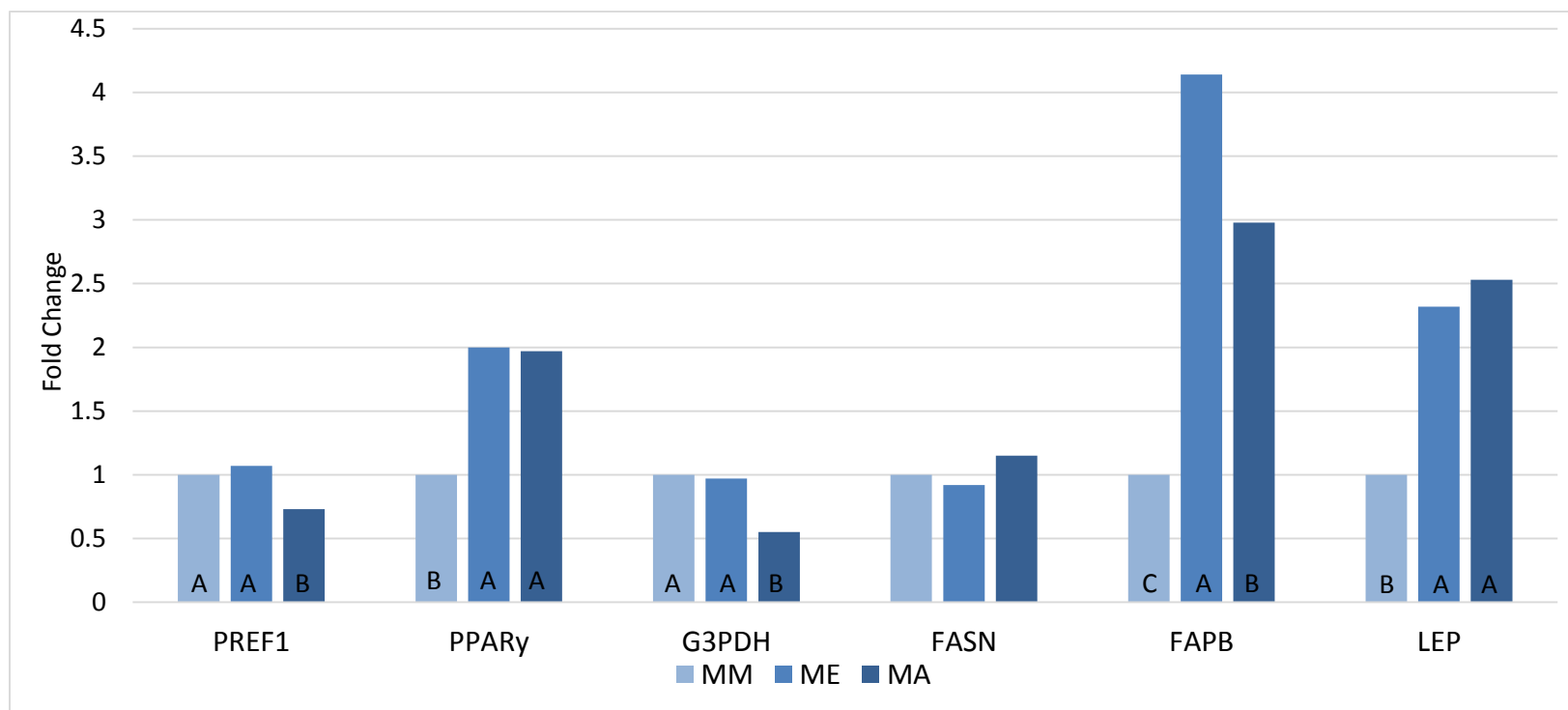
Table 5. Correlations of angiogenic gene expression in longissimus dorsi muscle (LM) with gene expression in corresponding immature, intermediate, and mature intramuscular adipose tissue (IM).

Item	VEGFA ¹ (LD)	ANGPT1 (LD)	ANGPT2 (LD)
<i>Immature IM</i>			
FASN	0.96*	0.91*	0.91*
PPAR γ	0.89*	0.91*	0.92*
ANGPT1	0.91*	0.94*	0.94*
ANGPT2	0.79*	0.69*	0.69*
ADIPOQ	0.93*	0.95*	0.96*
LEP	0.72*	0.56*	0.56
<i>Intermediate IM</i>			
FASN	0.01	-0.35	-0.75*
PPAR γ	-0.18	-0.20	-0.58*
ANGPT1	-0.19	-0.18	-0.53
ANGPT2	-0.15	-0.12	-0.35
ADIPOQ	-0.19	-0.43	-0.32
LEP	0.54	-0.18	-0.61*
<i>Mature IM</i>			
FASN	0.37	-0.06	-0.38
PPAR γ	0.37	0.18	-0.12
ANGPT1	0.42	0.26	-0.13
ANGPT2	-0.14	0.29	0.52
ADIPOQ	0.19	-0.22	-0.07
LEP	0.79*	-0.11	-0.43

¹ VEGFA=Vascular endothelial growth factor A; ANGPT1= Angiopoietin 1; ANGPT2= Angiopoietin 2; FASN= Fatty acid synthase; PPAR γ = Peroxisome proliferator activated receptor gamma; ADIPOQ= Adiponectin; LEP= Leptin.

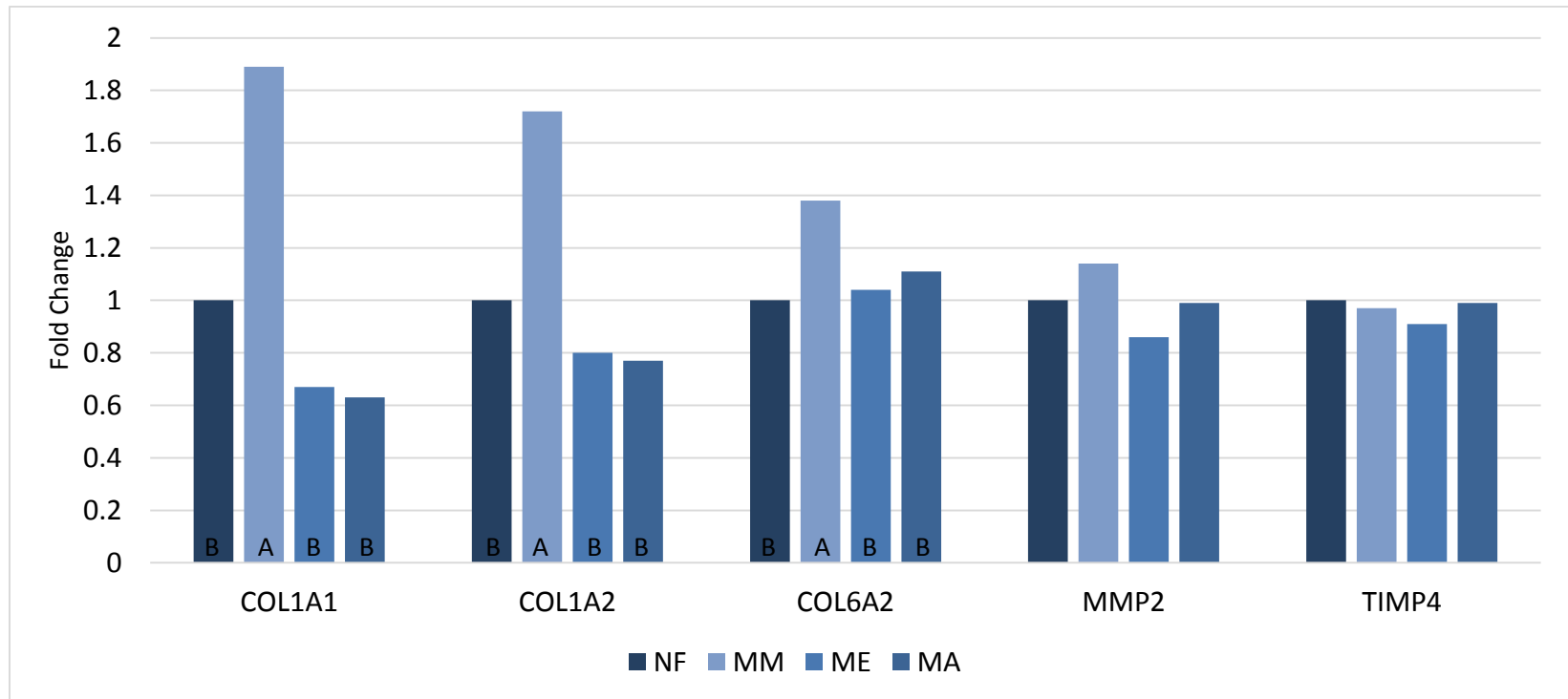
*Correlations are significantly different from zero (P < 0.05)

Figure 2. Relative expression of genes evaluated in IM tissue involved in adipogenesis.



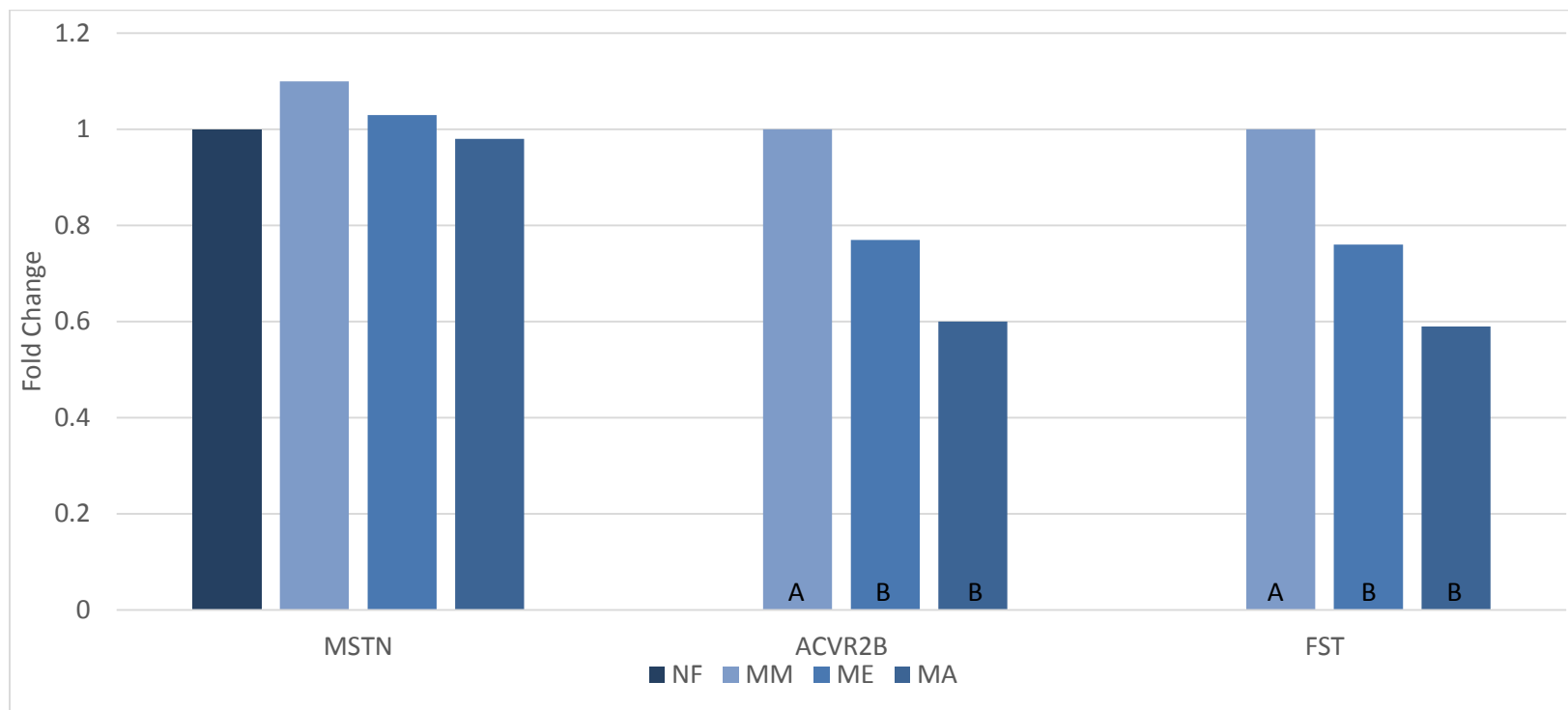
A,B,C means differ $P < 0.05$

Figure 3. Relative expression of genes evaluated in LM involved with remodeling of the extracellular matrix.



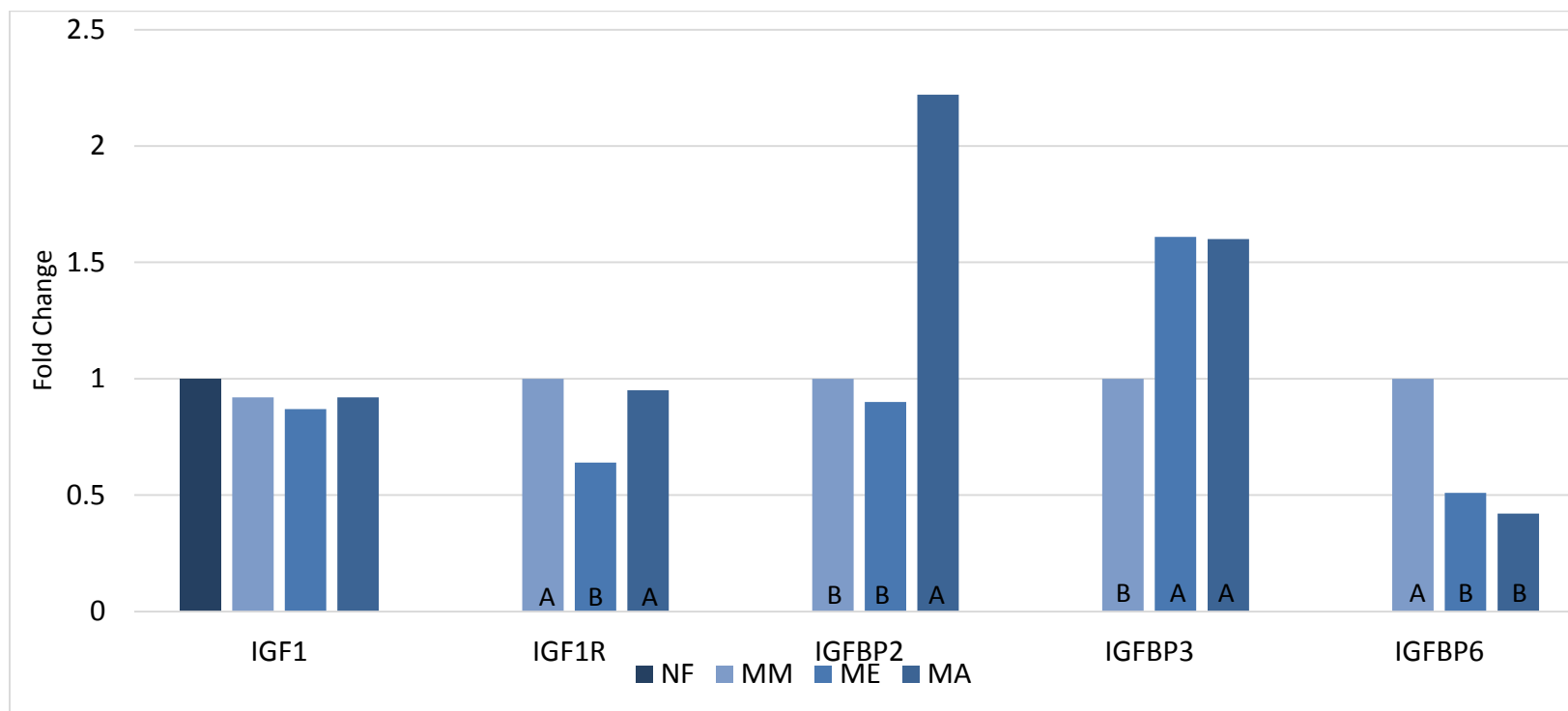
A, B means differ $P < 0.05$

Figure 4. Relative expression of genes involved in myostatin (MSTN) cell signaling. MSTN measured in LM tissue. ACVR2B and FST measured in IM tissue.



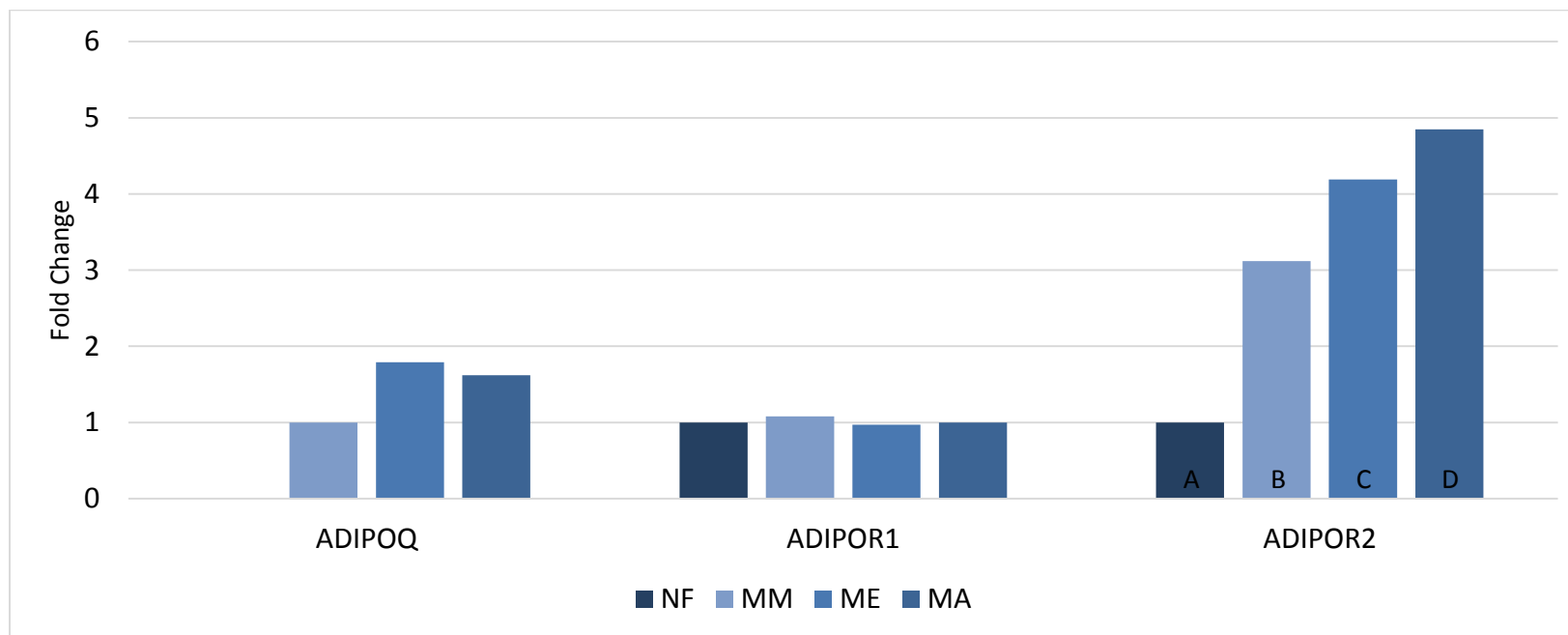
A, B means differ $P < 0.05$

Figure 5. Relative expression of genes involved in IGF-1 signaling pathways. IGF1 measured in LM tissue. IGF1R and IGFBPs measured in IM tissue.



A, B means differ $P < 0.05$

Figure 6. Relative expression of genes involved in the adiponectin signaling. ADIPOQ measured in IM tissue. ADIPOR1 and ADIPOR2 measured in LM tissue.



A, B, C, D means differ $P < 0.05$

CHAPTER V

CONCLUSION

Development of intramuscular fat is a complex process that is influenced by a variety of signals. The close proximity of intramuscular fat to muscle tissue during development indicates that intercellular signaling between these two tissues is crucial for development. This study shows that early in the development of intramuscular adipose tissue, remodeling of the extracellular matrix occurs along with angiogenesis is critical for development of intramuscular adipose tissue. The strong correlation between angiogenic growth factors in LM with angiogenic growth factors and markers of adipocyte differentiation in immature intramuscular fat development suggests that there is a highly coordinated change that occurs between skeletal muscle and intramuscular fat during the early stage of adipose development. This study also shows that skeletal muscle closely associated to intramuscular fat development may have increased sensitivity to insulin due to effects of adiponectin and leptin activity.

However, the mechanism of intramuscular adipose tissue regulation are still unclear and more in vitro and in vivo studies similar to this study need to be conducted to further elucidate the pathways and mechanism involved in intramuscular fat development. Further understanding the interactions between skeletal muscle and adipose tissue during intramuscular development could allow for development of management strategies that reduce waste fat and optimize carcass quality. Optimization of the development of muscle and adipose tissue will allow for the efficient production of high quality beef that will meet the demands of the consumer.

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VITA

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